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(54) Title: METHODS AND MEANS FOR ENHANCING SKIN TRANSPLANTATION USING GENE DELIVERY VEHICLES  
HAVING TROPISM FOR PRIMARY FIBROBLASTS, AS WELL AS OTHER USES THEREOF

(57) Abstract: The present invention relates to providing human primary fibroblasts with a nucleic acid of interest with, among others, the purpose to improve the taking of e.g. skin transplants. The invention particularly relates to transducing fibroblasts with said nucleic acid of interest by means of gene delivery vehicles, in particular chimaeric recombinant adenovirus (having an improved tropism for human primary fibroblasts) based gene delivery vehicles. The present invention is exemplified by an adenovirus serotype 5 genome based vector with an adenoviral fiber protein of a B-type or a D-type adenovirus, in particular adenovirus types 40 or 16, and wherein the nucleic acid of interest encodes a protein which improves angiogenesis and/or neurovascularisation, in particular myogenin or MyoD.

Title: Methods and means for enhancing skin transplantation using gene delivery vehicles having tropism for primary fibroblasts, as well as other uses thereof.

#### FIELD OF THE INVENTION

The present invention relates to the field of gene therapy. More in particular the invention relates to transducing fibroblasts with a nucleic acid of interest by means of adenovirus-based gene delivery vehicles.

5

#### BACKGROUND OF THE INVENTION

On a yearly basis, millions of people, worldwide, suffer from severe skin burns or chronic skin ulcers caused by pressure, venous status or diabetes mellitus (Brigham et al. 1996). Normal wound healing is a process that can be divided, schematically, into 3 phases (reviewed in Singer et al. 1999): 1) inflammation, 2) tissue formation, and 3) tissue remodeling. In the first phase, recruitment of platelets, leukocytes, neutrophils, and monocytes to the site of injury is pivotal to ensure the formation of an extra-cellular matrix that allows cell migration, cleansing of the wound of foreign particles, i.e. viruses, bacteria, and the initiation and propagation of new tissue formation. Some factors involved in initial recruitment of cells to a site of injury are the platelet derived growth factor (PDGF), transforming growth factor alpha and beta 1, 2, or 3 (TGF- $\alpha$ , TGF- $\beta$ 1, -2 and -3), fibroblast growth factor (FGF), Vascular endothelial growth factor (VEGF), insulin-like growth factor (IGF), or keratinocyte growth factor. Furthermore, other vasoactive and chemotactic factors secreted by injured parenchymal cells may play a role as well. In the second phase, epidermal cells at the wound margin start to proliferate into the wound space thereby

degrading the extra cellular matrix. Factors involved in these processes are collagenase 1, stromelysin 1, gelatinase A, collagenase 3 (matrix metalloproteinase 1, 3, 2, and 13) and plasmin produced by plasminogen activator (u-PA).

- 5 Proliferation of epidermal cells is driven by the release of many factors including epidermal growth factor (EGF), TGF- $\alpha$ , and keratinocyte growth factor (Pilcher et al. 1997; Bugge et al. 1996; Mignatti et al. 1996).

One critical factor in the process of re-  
10 epithelialization is neo-vascularization, since new blood vessels are required for the nutrient supply to the newly formed tissue. The induction of angiogenesis is a complex process involving many different molecules such as VEGF, TGF- $\beta$ , angiogenin, angiotropin, angiopoietin 1,  
15 thrombospondin, (Folkman et al. 1996; Iruela-Arispe et al. 1997; Risau 1997) and perhaps constitutively expressed nitric oxide synthase (ceNOS, ecNOS, NOSIII). The third phase involves the remodeling of collagen, appearance of myofibroblasts, wound contraction, and connective tissue  
20 compaction. In this phase of wound-healing again metalloproteinases, inhibitors of metalloproteinases play a crucial role. All in all, wound healing is a complex process involving many different steps, cell types, and stimulatory or inhibitory molecules. Abnormal healing of skin can be  
25 caused on any level described above. For instance, abnormalities in cell migration, proliferation, inflammation, synthesis and secretion of extra-cellular matrix proteins and cytokines, remodeling of wound matrix, increased activity of fibrogenic cytokines and exaggerated  
30 responses to these cytokines, mutations in regulatory genes such as p53, and abnormal epidermal-mesenchymal interactions have been reported (Fahey et al. 1991; Loots 1998, Tredget

et al. 1997; Babu et al. 1992, Zhang et al. 1995; Saed et al. 1998; Machesney et al. 1998).

For treatment of severe burn injuries or skin ulcers wound coverage is most important. Also, for plastic surgery, e.g. of the face, long-lasting skin grafts are required. Therefore, a variety of skin substitutes have been proposed each with its own advantages and disadvantages (see table 2). A major breakthrough in the field of tissue engineering of artificial skin has been the observation that there is no rejection of skin transplants using allogeneic epidermal cells. The lack of rejection is attributed to the absence of MHC-class II HLA-DR antigen expression on epidermal cells (Hefton et al. 1983) and the absence of Langerhans cells, the antigen presenting cells in the dermis (Thivolet et al. 1986). Thus, it is feasible to generate artificial skin for transplantation purposes to patients suffering from acute or chronic skin ulcers. The ingredients for the formation of artificial skin thus are a matrix providing attachment, fibroblasts and keratinocytes. The material of the matrix being collagen based dermal lattice. Since cultured epiderma-cell allografts are eventually replaced by host cells, their use is thought to be limited to temporary coverage of burns, skin-graft donor sites, and chronic open wounds such as pressure sores and venous stasis ulcers. Thus, it is thought that, unlike autologous grafts, allogeneic artificial skin is inappropriate for permanent coverage of full-thickness wounds. One of the reasons for the temporary use of artificial skin is the lack of take by the host. The grafting percentage of allogeneic artificial skin greatly depends on the material of which the matrix is constructed since the matrix may impose too great a diffusion barrier for the cultured cell graft to become vascularized. Unfortunately, regardless of the material

tested today, neovascularization of the skin graft is the most important factor limiting the use of allogeneic artificial skin transplantation.

The present invention now provides a solution for this  
5 limitation in that it provides methods and means to transduce human fibroblasts with viruses carrying genes encoding for proteins that promote angiogenesis such as described earlier. Preferably, the virus does not integrate into the host-cell genome since the desired effect should be  
10 transient only.

Gene-transfer vectors derived from adenoviruses (so-called adenoviral vectors) have a number of features that make them particularly useful for gene transfer: 1) the biology of the adenoviruses is characterized in detail, 2)  
15 the adenovirus is not associated with severe human pathology, 3) the virus is extremely efficient in introducing its DNA into the host cell, 4) the virus can infect a wide variety of cells and has a broad host-range, 5) the virus can be produced at high virus titers in large  
20 quantities, and 6) the virus can be rendered replication defective by deletion of the early-region 1 (E1) of the viral genome (Brody et al. 1994).

However, there are still drawbacks associated with the use of adenoviral vectors, especially the properly  
25 investigated serotypes of subgroup C adenoviruses. These serotypes require the presence of the Coxsackie adenovirus receptor (CAR) on cells for successful infection. Although this protein is expressed by many cells and established cell lines, this protein is absent on many other primary  
30 cells and cell lines making the latter cells difficult to infect with serotypes 1, 2, 5, and 6. Also pre-existing immunity and/or the immune response raised against the well known adenoviruses is another drawback.

The adenovirus genome is a linear double-stranded DNA molecule of approximately 36000 base pairs. The adenovirus DNA contains identical Inverted Terminal Repeats (ITR) of approximately 90-140 base pairs with the exact length depending on the serotype. The viral origins of replication are within the ITRs exactly at the genome ends. Most adenoviral vectors currently used in gene therapy have a deletion in the E1 region, where novel genetic information can be introduced. The E1 deletion renders the recombinant virus replication defective (Levrero et al. 1991). It has been demonstrated extensively that recombinant adenovirus, in particular serotype 5 is suitable for efficient transfer of genes *in vivo* to the liver, the airway epithelium and solid tumors in animal models and human xenografts in immunodeficient mice (Bout 1996; Blaese et al. 1995). Thus, preferred methods for *in vivo* gene transfer into target cells make use of adenoviral vectors as gene delivery vehicles.

At present, six different subgroups of human adenoviruses have been found which in total encompass 51 distinct adenovirus serotypes. Besides these human adenoviruses an extensive number of animal adenoviruses have been identified (see Ishibashi et al. 1983). A serotype is defined on the basis of its immunological distinctiveness as determined by quantitative neutralization with animal antisera (horse, rabbit). If neutralization shows a certain degree of cross-reaction between two viruses, distinctiveness of serotype is assumed if A) the hemagglutinins are unrelated, as shown by lack of cross-reaction on hemagglutination-inhibition, or B) substantial biophysical/ biochemical differences in DNA exist (Francki et al. 1991). The nine serotypes identified

last (42-51) were isolated for the first time from HIV-infected patients (Hierholzer et al. 1988; Schnurr et al. 1993). For reasons not well understood, most of such immuno-compromised patients shed adenoviruses that were rarely or never isolated from immuno-competent individuals (Hierholzer et al. 1988, 1992; Khoo et al. 1995).

The adenovirus serotype 5 (Ad5) is most widely used for gene therapy purposes. Similar to serotypes 2, 4 and 7, serotype 5 has a natural affiliation towards lung epithelia and other respiratory tissues. In contrast, it is known that, for instance, serotypes 40 and 41 have a natural affiliation towards the gastrointestinal tract. For a detailed overview of the disease association of the different adenovirus serotypes see table 1. In this table there is one deviation from the literature. Sequence analysis and hemagglutination assays using erythrocytes from different species performed in our institute indicated that in contrast to the literature (De Jong et al. 1999) adenovirus 50 proved to be a D group vector whereas adenovirus 51 proved to be a B-group vector. The natural affiliation of a given serotype towards a specific organ can either be due to a difference in the route of infection i.e. make use of different receptor molecules or internalization pathways. However, it can also be due to the fact that a serotype can infect many tissues/organs but it can only replicate in one organ because of the requirement of certain cellular factors for replication and hence clinical disease. At present it is unknown which of the above mentioned mechanisms is responsible for the observed differences in human disease association. However it is known that different adenovirus serotypes can bind to different receptors due to sequence dissimilarity of the capsid proteins i.e. hexon, penton, and fiber protein. For

instance, it has been shown that adenoviruses of subgroup C such as Ad2, and Ad5 bind to different receptors as compared to adenoviruses from subgroup B such as Ad3 (Defer et al. 1990). Likewise, it was demonstrated that receptor  
5 specificity could be altered by exchanging the Ad3 with the Ad5 knob protein, and vice versa (Krasnykh et al. 1996; Stevenson et al. 1995 and 1997). The present invention now applies this knowledge to identify gene delivery vehicles that can be put to a novel and inventive use in transducing  
10 primary fibroblasts which are used to improve skin grafting and in other medicinal applications.

Next, the steps involved in adenovirus binding as has been elucidated at present will be disclosed. The initial  
15 step for successful infection is binding of adenovirus to its target cell, a process mediated through fiber protein. The fiber protein has a trimeric structure (Stouten et al. 1992) with different lengths depending on the virus serotype (Signas et al. 1985; Kidd et al. 1993). Different  
20 serotypes have polypeptides with structurally similar N- and C-termini, but different middle stem regions. At the N-terminus, the first 30 amino acids are involved in anchoring of the fiber to the penton base (Chroboczek et al. 1995), especially the conserved FNPVYP region in the  
25 tail (Arnberg et al. 1997). The C-terminus, or knob, is responsible for initial interaction with the cellular adenovirus receptor. After this initial binding secondary binding between the capsid penton base and cell-surface integrins is proposed to lead to internalization of viral  
30 particles in coated pits and endocytosis (Morgan et al. 1969; Svensson et al. 1984; Varga et al. 1992; Greber et al. 1993; Wickham et al. 1994). Integrins are  $\alpha\beta$ -heterodimers of which at least 14  $\alpha$ -subunits and 8  $\beta$ -



subunits have been identified (Hynes et al. 1992). The array of integrins expressed in cells is complex and will vary between cell types and cellular environment. Although the knob contains some conserved regions, between

5 serotypes, knob proteins show a high degree of variability, indicating that different adenovirus receptors might exist. For instance, it has been demonstrated that adenoviruses of subgroup C (Ad2, Ad5) and adenoviruses of subgroup B (Ad3) bind to different receptors (Defner et al. 1990). By using

10 baculovirus produced soluble CAR as well as adenovirus serotype 5 knob protein, Roelvink et al. (1998) concluded via interference studies that all adenovirus serotypes, except serotypes of subgroup B, enter cells via CAR. The latter, if valid limits the complexity of using different

15 serotypes for gene therapy purposes.

Besides the involvement in cell binding, the fiber protein also contains the type specific  $\gamma$ -antigen, which together with the  $\epsilon$ -antigen of the hexon determines the serotype specificity. The  $\gamma$ -antigen is localized on the

20 fiber and it is known that it consists of 17 aminoacids (Eiz et al. 1997). The anti-fiber antibodies of the host are therefore directed to the trimeric structure of the knob. To obtain re-directed infection of recombinant adenovirus serotype 5, several approaches have been or

25 still are under investigation. Wickham et al. (1995 and 1996) have altered the RGD (Arg, Gly, Asp) motif in the penton base which is believed to be responsible for the  $\alpha_v\beta_3$  and  $\alpha_v\beta_5$  integrin binding to the penton base. They have replaced this RGD motif by another peptide motif which is

30 specific for the  $\alpha_4\beta_1$  receptor. In this way targeting the adenovirus to a specific target cell could be accomplished. Krasnykh and colleagues (1998) have made use of the HI loop available in the knob. This loop is, based on X-ray

crystallographics, located on the outside of the knob trimeric structure and therefore is thought not to contribute to the intra molecular interactions in the knob. Insertion of a FLAG coding sequence into the HI loop  
5 resulted in targeting of the adenovirus to target cells by using antibodies which recognize both the FLAG epitope and a cellular receptor. However, complete CAR independent infection was not observed.

The invention provides the use of a recombinant  
10 adenovirus having a tropism for human primary fibroblasts as a vehicle for delivering a nucleic acid of interest to a human primary fibroblast. The tropism for the (human) primary fibroblast may be a tropism that the virus itself exhibits, or preferably a tropism that the virus has been  
15 provided with. As mentioned herein before the tropism is at least partially accounted for by the fiber protein of a (recombinant) adenovirus. Typically the adenovirus of the invention will therefor have at least a tropism determining part of a fiber protein of a virus fiber protein having  
20 tropism for primary fibroblasts. In this is not the wild type fiber protein of the adenovirus used, the result is a chimaeric adenovirus. The invention thus also provides a use in which said recombinant adenovirus is a chimaeric adenovirus. We have shown a number of fibers, which when  
25 grafted in an adenovirus 5 (the sequence encoding the fiber replaces the ad5 sequence encoding the fiber), typically fibers or parts thereof derived from group B and/or D viruses to have a higher infection rate of human primary fibroblasts. Thus the invention provides viruses and their  
30 use, wherein said tropism is provided by at least a tropism determining part of an adenoviral fiber protein of a B-type or a D-type adenovirus, in particular from an adenovirus type 11, 16, 35, 51, 9, 13, 17, 32 and/or 38. Of course

combinations of motifs from these fibers in and Ad5 backbone or in another backbone, in particular one with reduced immunogenicity (in any case a preferred embodiment of the present invention) are also part of the present invention. This also goes for a particular fiber from adenovirus type 40. Thus the invention also provides a virus and its use, wherein said fiber protein is derived from a short fiber protein of an adenovirus type 40. The most preferred virus for transfecting primary fibroblasts is one wherein the tropism is derived from a fiber protein of an adenovirus type 16 or a functional equivalent and/or homologue thereof. A functional equivalent is an amino acid sequence based on or derived from the fiber 16 sequence, which has the same function (in kind, not necessarily in quantity) of providing said tropism. A homologue is an amino acid sequence derived from a different virus, but having the same or similar function as the fiber 16 sequence. Typically high homology between the equivalent and/or the homologue and the original fiber 16 sequence will exist. One of the objectives of the present invention is to improve methods of skin transplatation (temporarily and/or permanently) by means of gene therapy to primary fibroblasts in a skin graft. For that purpose it is preferred to provide the gene delivery vehicles with a nucleic acid of interest encoding a proteinaceous substance which improves angiogenesis and/or neovascularisation, since this is one of the main problems for such grafts.

Combinations of different angiogenic proteins are of course also part of the present invention. A proteinaceous substance is a substance which comprises amino acids linked through a peptide bond, such as (poly)peptides, proteins, glycosylated polypeptides, proteins comprising subunits and complexes of proteins with nucleic acids.

The invention also provides the use of gene delivery vehicles according to the invention to transduce primary fibroblasts with nucleic acids of interest for different syndromes. Thus the invention provides a gene delivery  
5 vehicle and its use wherein said nucleic acid of interest is a MyoD gene and/or a Myogenin gene or a functional equivalent thereof. Another area in which efficient adenoviral transduction of primary human fibroblasts is a prerequisite is genetic counseling for couples that are  
10 suspected of inherited disease, an example being Duchenne Muscular Dystrophy (DMD). On a yearly basis, thousands of couples request family screening for DMD when this disease is known to 'run in the family'. The cells of choice for testing are primary human fibroblasts obtained from skin  
15 biopsies. The primary fibroblasts are expanded and used for PCR-based mutational analysis. In hundreds of cases, PCR-analysis is not conclusive and differentiation of primary fibroblasts to myofibroblasts is required (Roest et al. 1996a and 1996b). The myofibroblasts may or may not express  
20 the dystrophin protein that can be detected using standard immuno histological methods. To ensure a rapid phenotypic differentiation of fibroblasts to myofibroblasts, the MyoD gene and/or the Myogenin gene need to be introduced into fibroblasts. This has proven difficult with Ad5 based  
25 adenovirus since only small percentages can be transduced of which only a small percentage differentiates into myofibroblasts presumably due to low levels of expression of the MyoD and/or Myogenin gene.

Since adenovirus 5 is a well known virus with  
30 promising results in the field of gene therapy so far, one of the preferred embodiments of the present invention is to provide a recombinant adenovirus based on type 5, with a nucleic acid of interest inserted therein and the fiber

replaced by a fiber (or part thereof) having the desired tropism.

Thus the invention also provides a virus and its use wherein said virus comprises an adenovirus 5 nucleic acid sequence, preferably a chimaeric virus having at least a deletion in its E1 region where a nucleic acid of interest is inserted or can be inserted and a deletion in the fiber region which is replaced by a nucleic acid sequence having the desired tropism. Apart from the uses described above the invention also provides the recombinant adenoviruses as described above, herein also designated as gene delivery vehicles. However gene delivery vehicles according to the invention are based on adenovirus (for tropism in particular) but may comprise parts of other viruses, even to the extent that only the tropism is derived from an adenoviral fiber. Preferred are chimaeric adenoviruses, which also include chimaeras with other viruses such as AAV, retroviruses, etc.

Thus the invention provides in a preferred embodiment a gene delivery vehicle for delivering a nucleic acid of interest to a primary fibroblast, comprising a recombinant adenovirus having tropism for a primary fibroblast and a nucleic acid sequence encoding a proteinaceous substance which improves angiogenesis and/or neovascularisation as a nucleic acid of interest.

In a further preferred embodiment the invention provides a gene delivery vehicle for delivering a nucleic acid of interest to a primary fibroblast, comprising a recombinant adenovirus having tropism for a primary fibroblast and a MyoD gene and/or a Myogenin gene or a functional equivalent thereof. Preferably said tropism for the gene delivery vehicles according to the invention is provided by a nucleic acid sequence encoding at least a

tropism determining part of a fiber protein of an adenovirus type B and/or adenovirus type D, in particular by a nucleic acid sequence encoding at least a tropism determining part of a fiber protein of an adenovirus type  
5 11, 16, 35, 51, 9, 13, 17, 32 and/or 38.

Most preferred is a gene delivery vehicle wherein said tropism is provided by a nucleic acid sequence encoding at least a tropism determining part of a fiber protein of an adenovirus type 16.

10 As stated herein before a highly preferred gene delivery vehicle is one wherein said chimaeric virus comprises an adenovirus 5 genome, having at least a deletion in its E1 region where a nucleic acid of interest is inserted or can be inserted and a deletion in the fiber  
15 region which is replaced by a nucleic acid sequence having the desired tropism. Preferably further alterations are made to decrease immunogenicity (providing parts of other adenoviruses such as Ad35 or equivalents thereof).

Cells for e.g. skin allografts are also part of the  
20 present invention, which provides a human primary fibroblast transduced with a gene delivery vehicle according to the invention. Skin allograft comprising a human primary fibroblast described above or offspring thereof are also part of the present invention. The invention further  
25 provides a method for improving allogeneic or autologous skin transplantation comprising transducing human primary fibroblasts with a gene delivery vehicle according to the invention, preparing a skin graft with said human primary fibroblasts and applying said graft to a patient.

30 The invention uses a library of adenoviruses in which the sequence encoding for the fiber protein from alternative serotypes has been cloned into an adenovirus serotype 5 backbone thereby generating a chimaeric

adenovirus. This chimaeric adenovirus thus has the host range of the adenovirus serotype of which the fiber sequence was cloned whereas all other aspects are derived from adenovirus serotype 5. Of course also a gene of interest can be inserted at for instance the site of E1 of the original adenovirus from which the vector is derived. In this manner the chimaeric adenovirus to be produced can be adapted to the requirements and needs of certain hosts in need of gene therapy for certain disorders. Naturally, to enable production of a chimaeric adenovirus, a packaging cell will generally be needed in order to produce sufficient amount of safe chimaeric adenoviruses.

An important feature of the present invention is the means to produce the chimaeric virus. Typically, one does not want an adenovirus batch that contains replication competent adenovirus to be administered to a host cell, although this is not always true. In general therefore it is desired to omit a number of genes (but at least one) from the adenoviral genome on the vector encoding the chimaeric virus and to supply these genes in the genome of the cell in which the vector is brought to produce chimaeric adenovirus. Such a cell is usually called a packaging cell. The invention thus also provides a packaging cell for producing a chimaeric adenovirus according to the invention, comprising in trans all elements necessary for adenovirus production not present on the adenoviral vector according to the invention. Typically vector and packaging cell have to be adapted to one another in that they have all the necessary elements, but that they do not have overlapping elements which lead to replication competent virus by homologous recombination.

The present invention provides methods and means by which an adenovirus can infect primary human fibroblasts. Therefore, the generation of chimaeric adenoviruses based on adenovirus serotype 5 (Ad5) with modified fiber genes is disclosed. For this purpose, two or three plasmids, which together contain the complete adenovirus serotype 5 genome, were constructed. From this plasmid the DNA encoding the adenovirus serotype 5 fiber protein was removed and replaced by linker DNA sequences that facilitate easy cloning. The plasmid in which the native adenovirus serotype 5 fiber sequence was partially removed subsequently served as template for the insertion of DNA encoding for fiber protein derived from different adenovirus serotypes (human or animal). The DNA's derived from the different serotypes were obtained using the polymerase chain reaction (PCR) technique in combination with (degenerated) oligo-nucleotides. At the former E1 location in the genome of adenovirus serotype 5, any gene of interest can be cloned. A single transfection procedure of the two or three plasmids together resulted in the formation of a recombinant chimaeric adenovirus. Although successful introduction of changes in the adenovirus serotype 5 fiber and penton-base have been reported by others, the complex structure of knob and the limited knowledge of the precise amino acids interacting with CAR render such targeting approaches laborious and difficult.

To overcome the limitations described above we used pre-existing adenovirus fibers to maximize the chance of obtaining recombinant adenovirus which can normally assemble in the nucleus of a producer cell and which can be produced on pre-existing packaging cells. By generating a chimaeric adenovirus serotype 5 based fiber library containing fiber proteins of all other human adenovirus



serotypes, we have developed a technology which enables rapid screening for a recombinant adenoviral vector with preferred infection characteristics for primary human fibroblasts.

5        In one aspect the invention describes the construction and use of plasmids consisting of distinct parts of adenovirus serotype 5 in which the gene encoding for fiber protein has been replaced with DNA derived from alternative human or animal serotypes. This set of constructs, in total  
10 encompassing the complete adenovirus genome, allows for the construction of unique chimaeric adenoviruses customized for transduction of particular cell types or organ(s). Also, in this part of the invention means and methods to propagate, produce, and purify fiber chimaeric adenoviruses  
15 are disclosed.

      In another aspect of the invention chimaeric viruses are described which have preferred infection characteristics in human primary fibroblasts. The adenoviral vectors preferably are derived from subgroup B  
20 adenoviruses or contain at least a functional part of the fiber protein from an adenovirus from subgroup B comprising at least the binding moiety of the fiber protein. In a further preferred embodiment the adenoviral vectors are chimaeric vectors based on adenovirus serotype 5 and  
25 contain at least a functional part of the fiber protein from adenovirus type 16, 35, or 51. It is to be understood that in all embodiments the adenoviral vectors may be derived from the serotype having the desired properties or that the adenoviral vector is based on an adenovirus from  
30 one serotype and contains the sequences comprising the desired functions of another serotype, these sequences replacing the native sequences in the said serotype

In another aspect of the invention the chimaeric adenoviruses may, or may not, contain deletions in the E1 region and insertions of heterologous genes linked either or not to a promoter. Furthermore, chimaeric adenoviruses  
5 may, or may not, contain deletions in the E3 region and insertions of heterologous genes linked to a promoter. Furthermore, chimaeric adenoviruses may, or may not, contain deletions in the E2 and/ or E4 region and insertions of heterologous genes linked to a promoter. In  
10 the latter case E2 and/ or E4 complementing cell lines are required to generate recombinant adenoviruses.

## EXAMPLES

To illustrate the invention, the following examples are provided, not intended to limit the scope of the invention.

5

**Example 1****Generation of adenovirus serotype 5 genomic plasmid clones**

The complete genome of adenovirus serotype 5 has been cloned into various plasmids or cosmids to allow easy  
10 modification of parts of the adenovirus serotype 5 genome, while still retaining the capability to produce recombinant virus. For this purpose the following plasmids were generated:

15 pBr/Ad.Bam-rITR (ECACC deposit P97082122)

In order to facilitate blunt end cloning of the ITR sequences, wild-type human adenovirus type 5 (Ad5) DNA was treated with Klenow enzyme in the presence of excess dNTPs. After inactivation of the Klenow enzyme and purification by  
20 phenol/chloroform extraction followed by ethanol precipitation, the DNA was digested with BamHI. This DNA preparation was used without further purification in a ligation reaction with pBr322 derived vector DNA prepared as follows: pBr322 DNA was digested with EcoRV and BamHI,  
25 dephosphorylated by treatment with TSAP enzyme (Life Technologies) and purified on LMP agarose gel (SeaPlaque GTG). After transformation into competent *E.coli* DH5 $\alpha$  (Life Techn.) and analysis of ampicillin resistant colonies, one clone was selected that showed a digestion pattern as  
30 expected for an insert extending from the BamHI site in Ad5 to the right ITR.

Sequence analysis of the cloning border at the right ITR revealed that the most 3' G residue of the ITR was missing,

the remainder of the ITR was found to be correct. Said missing G residue is complemented by the other ITR during replication.

5 pBr/Ad.Sal-rITR (ECACC deposit P97082119)

pBr/Ad.Bam-rITR was digested with BamHI and SalI. The vector fragment including the adenovirus insert was isolated in LMP agarose (SeaPlaque GTG) and ligated to a 4.8 kb SalI-BamHI fragment obtained from wt Ad5 DNA and purified with  
10 the Geneclean II kit (Bio 101, Inc.). One clone was chosen and the integrity of the Ad5 sequences was determined by restriction enzyme analysis. Clone pBr/Ad.Sal-rITR contains adeno type 5 sequences from the SalI site at bp 16746 up to and including the rITR (missing the most 3' G residue).

15

pBr/Ad.Cla-Bam (ECACC deposit P97082117)

Wild type adenovirus type 5 DNA was digested with ClaI and BamHI, and the 20.6 kb fragment was isolated from gel by electro-elution. pBr322 was digested with the same enzymes  
20 and purified from agarose gel by Geneclean. Both fragments were ligated and transformed into competent DH5 $\alpha$ . The resulting clone pBr/Ad.Cla-Bam was analyzed by restriction enzyme digestion and shown to contain an insert with adenovirus sequences from bp 919 to 21566.

25

pBr/Ad.AflIII-Bam (ECACC deposit P97082114)

Clone pBr/Ad.Cla-Bam was linearized with EcoRI (in pBr322) and partially digested with AflIII. After heat inactivation of AflIII for 20 min at 65°C the fragment ends  
30 were filled in with Klenow enzyme. The DNA was then ligated to a blunt double stranded oligo linker containing a PacI site (5'-AATTGTCTTAATTAACCGCTTAA-3'). This linker was made by annealing the following two oligonucleotides: 5'-

AATTGTCTTAATTAACCGC-3' and 5'-AATTGCGGTTAATTAAGAC-3', followed by blunting with Klenow enzyme. After precipitation of the ligated DNA to change buffer, the ligations were digested with an excess PacI enzyme to remove concatameres of the oligo. The 22016 bp partial fragment containing Ad5 sequences from bp 3534 up to 21566 and the vector sequences, was isolated in LMP agarose (SeaPlaque GTG), religated and transformed into competent DH5 $\alpha$ . One clone that was found to contain the PacI site and that had retained the large adeno fragment was selected and sequenced at the 5' end to verify correct insertion of the PacI linker in the (lost) AflIII site.

pBr/Ad.Bam-rITRpac#2 (ECACC deposit P97082120) and  
15 pBr/Ad.Bam-rITR#8 (ECACC deposit P97082121)

To allow insertion of a PacI site near the ITR of Ad5 in clone pBr/Ad.Bam-rITR about 190 nucleotides were removed between the ClaI site in the pBr322 backbone and the start of the ITR sequences. This was done as follows: pBr/Ad.Bam-rITR was digested with ClaI and treated with nuclease Bal31 for varying lengths of time (2', 5', 10' and 15'). The extent of nucleotide removal was followed by separate reactions on pBr322 DNA (also digested at the ClaI site), using identical buffers and conditions. Bal31 enzyme was inactivated by incubation at 75°C for 10 minutes, the DNA was precipitated and resuspended in a smaller volume of TE buffer. To ensure blunt ends, DNAs were further treated with T4 DNA polymerase in the presence of excess dNTPs. After digestion of the (control) pBr322 DNA with SalI, satisfactory degradation (~150 bp) was observed in the samples treated for 10 min or 15 min. The 10 min or 15 min treated pBr/Ad.Bam-rITR samples were then ligated to the above described blunted PacI linkers (See pBr/Ad.AflIII-Bam).

Ligations were purified by precipitation, digested with excess PacI and separated from the linkers on an LMP agarose gel. After religation, DNAs were transformed into competent DH5a and colonies analyzed. Ten clones were selected that  
5 showed a deletion of approximately the desired length and these were further analyzed by T-track sequencing (T7 sequencing kit, Pharmacia Biotech). Two clones were found with the PacI linker inserted just downstream of the rITR. After digestion with PacI, clone #2 has 28 bp and clone #8  
10 has 27 bp attached to the ITR.

pWE/Ad.AflIII-rITR (ECACC deposit P97082116)

Cosmid vector pWE15 (Clontech) was used to clone larger Ad5 inserts. First, a linker containing a unique PacI site  
15 was inserted in the EcoRI sites of pWE15 creating pWE.pac. To this end, the double stranded PacI oligo as described for pBr/Ad.AflIII-BamHI was used but now with its EcoRI protruding ends. The following fragments were then isolated by electro-elution from agarose gel: pWE.pac digested with  
20 PacI, pBr/AflIII-Bam digested with PacI and BamHI and pBr/Ad.Bam-rITR#2 digested with BamHI and PacI. These fragments were ligated together and packaged using 1 phage packaging extracts (Stratagene) according to the manufacturer's protocol. After infection into host bacteria,  
25 colonies were grown on plates and analyzed for presence of the complete insert. pWE/Ad.AflIII-rITR contains all adenovirus type 5 sequences from bp 3534 (AflIII site) up to and including the right ITR (missing the most 3' G residue).

30 pBr/Ad.lITR-Sal(9.4) (ECACC deposit P97082115)

Adenovirus 5 wild type DNA was treated with Klenow enzyme in the presence of excess dNTPs and subsequently digested with SalI. Two of the resulting fragments,

designated left ITR-Sal(9.4) and Sal(16.7)-right ITR, respectively, were isolated in LMP agarose (Seaplaque GTG). pBr322 DNA was digested with EcoRV and SalI and treated with phosphatase (Life Technologies). The vector fragment was  
5 isolated using the Geneclean method (BIO 101, Inc.) and ligated to the Ad5 SalI fragments. Only the ligation with the 9.4 kb fragment gave colonies with an insert. After analysis and sequencing of the cloning border a clone was chosen that contained the full ITR sequence and extended to  
10 the SalI site at bp 9462.

pBr/Ad.1ITR-Sal(16.7) (ECACC deposit P97082118)

pBr/Ad.1ITR-Sal(9.4) is digested with SalI and dephosphorylated (TSAP, Life Technologies). To extend this  
15 clone upto the third SalI site in Ad5, pBr/Ad.Cla-Bam was linearized with BamHI and partially digested with SalI. A 7.3 kb SalI fragment containing adenovirus sequences from 9462-16746 was isolated in LMP agarose gel and ligated to the SalI-digested pBr/Ad.1ITR-Sal(9.4) vector fragment.

20

pWE/Ad.AflIII-EcoRI

pWE.pac was digested with ClaI and 5' protruding ends were filled using Klenow enzyme. The DNA was then digested with PacI and isolated from agarose gel. pWE/AflIII-rITR was  
25 digested with EcoRI and after treatment with Klenow enzyme digested with PacI. The large 24 kb fragment containing the adenoviral sequences was isolated from agarose gel and ligated to the ClaI-digested and blunted pWE.pac vector using the Ligation Express™ kit (Clontech). After  
30 transformation of Ultracompetent XL10-Gold cells (Stratagene), clones were identified that contained the expected insert. pWE/AflIII-EcoRI contains Ad5 sequences from bp 3534-27336.

Construction of new adapter plasmids

The absence of sequence overlap between the recombinant adenovirus and E1 sequences in the packaging cell line is essential for safe, RCA-free generation and propagation of new recombinant viruses. The adapter plasmid pMLPI.TK is an example of an adapter plasmid designed for use according to the invention in combination with the improved packaging cell lines of the invention. This plasmid was used as the starting material to make a new vector in which nucleic acid molecules comprising specific promoter and gene sequences can be easily exchanged. First, a PCR fragment was generated from pZipΔMo+PyF101(N<sup>-</sup>) template DNA (described in WO 96/35798) with the following primers: LTR-1: 5'-CTG TAC GTA CCA GTG CAC TGG CCT AGG CAT GGA AAA ATA CAT AAC TG-3' and LTR-2: 5'-GCG GAT CCT TCG AAC CAT GGT AAG CTT GGT ACC GCT AGC GTT AAC CGG GCG ACT CAG TCA ATC G-3'. Pwo DNA polymerase (Boehringer Mannheim) was used according to manufacturers protocol with the following temperature cycles: once 5 min at 95°C; 3 min at 55°C; and 1 min at 72°C, and 30 cycles of 1 min at 95°C, 1 min at 60°C, 1 min at 72°C, followed by once 10 min at 72°C. The PCR product was then digested with BamHI and ligated into pMLP10 (Levrero et al. 1991) vector digested with PvuII and BamHI, thereby generating vector pLTR10. This vector contains adenoviral sequences from bp 1 up to bp 454 followed by a promoter consisting of a part of the Mo-MuLV LTR having its wild-type enhancer sequences replaced by the enhancer from a mutant polyoma virus (PyF101). The promoter fragment was designated L420. Next, the coding region of the murine HSA gene was inserted. pLTR10 was digested with BstBI followed by Klenow treatment and digestion with NcoI. The HSA gene was obtained by PCR amplification on pUC18-HSA (Kay et al. 1990) using the



following primers: HSA1, 5'-GCG CCA CCA TGG GCA GAG CGA TGG  
TGG C-3' and HSA2, 5'-GTT AGA TCT AAG CTT GTC GAC ATC GAT  
CTA CTA ACA GTA GAG ATG TAG AA-3'. The 269 bp amplified  
5 and BglII sites. Sequencing confirmed incorporation of the  
correct coding sequence of the HSA gene, but with an extra  
TAG insertion directly following the TAG stop codon. The  
coding region of the HSA gene, including the TAG duplication  
was then excised as a NcoI (sticky)-SalI (blunt) fragment  
10 and cloned into the 3.5 kb NcoI(sticky)/BstBI(blunt)  
fragment from pLTR10, resulting in pLTR-HSA10.

Finally, pLTR-HSA10 was digested with EcoRI and BamHI  
after which the fragment containing the left ITR, packaging  
signal, L420 promoter and HSA gene was inserted into vector  
15 pMLPI.TK digested with the same enzymes and thereby  
replacing the promoter and gene sequences. This resulted in  
the new adapter plasmid pAd/L420-HSA that contains  
convenient recognition sites for various restriction enzymes  
around the promoter and gene sequences. SnaBI and AvrII can  
20 be combined with HpaI, NheI, KpnI, HindIII to exchange  
promoter sequences, while the latter sites can be combined  
with the ClaI or BamHI sites 3' from HSA coding region to  
replace genes in this construct.

Another adapter plasmid that was designed to allow easy  
25 exchange of nucleic acid molecules was made by replacing the  
promoter, gene and poly A sequences in pAd/L420-HSA with the  
CMV promoter, a multiple cloning site, an intron and a poly-  
A signal. For this purpose, pAd/L420-HSA was digested with  
AvrII and BglII followed by treatment with Klenow to obtain  
30 blunt ends. The 5.1 kb fragment with pBr322 vector and  
adenoviral sequences was isolated and ligated to a blunt  
1570 bp fragment from pcDNA1/amp (Invitrogen) obtained by

digestion with HhaI and AvrII followed by treatment with T4 DNA polymerase. This adapter plasmid was named pCLIP.

#### Generation of recombinant adenoviruses

- 5        To generate E1 deleted recombinant adenoviruses with the new plasmid-based system, the following constructs are prepared:
- a) An adapter construct containing the expression cassette with the gene of interest linearized with a restriction  
10 enzyme that cuts at the 3' side of the overlapping adenoviral genome fragment, preferably not containing any pBr322 vector sequences, and
- b) A complementing adenoviral genome construct pWE/Ad.AflIII-rITR digested with PacI.
- 15        These two DNA molecules are further purified by phenol/chloroform extraction and Ethanol precipitation. Co-transfection of these plasmids into an adenovirus packaging cell line, preferably a cell line according to the invention, generates recombinant replication deficient  
20 adenoviruses by a one-step homologous recombination between the adapter and the complementing construct.

Alternatively, instead of pWE/Ad.AflIII-rITR other fragments can be used, e.g., pBr/Ad.Cla-Bam digested with EcoRI and BamHI or pBr/Ad.AflIII-BamHI digested with PacI and  
25 BamHI can be combined with pBr/Ad.Sal-rITR digested with SalI. In this case, three plasmids are combined and two homologous recombinations are needed to obtain a recombinant adenovirus. It is to be understood that those skilled in the art may use other combinations of adapter and complementing  
30 plasmids without departing from the present invention.

A general protocol as outlined below and meant as a non-limiting example of the present invention has been performed to produce several recombinant adenoviruses using

various adapter plasmids and the Ad.AflIII-rITR fragment. Adenovirus packaging cells (PER.C6) were seeded in ~25 cm<sup>2</sup> flasks and the next day when they were at ~80% confluency, transfected with a mixture of DNA and lipofectamine agent (Life Techn.) as described by the manufacturer. Routinely, 40 µl lipofectamine, 4 µg adapter plasmid and 4 µg of the complementing adenovirus genome fragment AflIII- rITR (or 2 µg of all three plasmids for the double homologous recombination) are used. Under these conditions transient transfection efficiencies of ~50% (48 hrs post transfection) are obtained as determined with control transfections using a pAd/CMV-LacZ adapter. Two days later, cells are passaged to ~80 cm<sup>2</sup> flasks and further cultured. Approximately five (for the single homologous recombination) to eleven days (for the double homologous recombination) later a cytopathogenic effect (CPE) is seen, indicating that functional adenovirus has formed. Cells and medium are harvested upon full CPE and recombinant virus is released by freeze-thawing. An extra amplification step in an 80 cm<sup>2</sup> flask is routinely performed to increase the yield since at the initial stage the titers are found to be variable despite the occurrence of full CPE. After amplification, viruses are harvested and plaque purified on PER.C6 cells. Individual plaques are tested for viruses with active transgenes.

Besides replacements in the E1 region it is possible to delete or replace (part of) the E3 region in the adenovirus because E3 functions are not necessary for the replication, packaging and infection of the (recombinant) virus. This creates the opportunity to use a larger insert or to insert more than one gene without exceeding the maximum package size (approximately 105% of wt genome length). This can be done, e.g., by deleting part of the E3 region in the pBr/Ad.Bam-rITR clone by digestion with XbaI and religation.

This removes Ad5 wt sequences 28592-30470 including all known E3 coding regions. Another example is the precise replacement of the coding region of gp19K in the E3 region with a polylinker allowing insertion of new sequences. This, 5 1) leaves all other coding regions intact and 2) obviates the need for a heterologous promoter since the transgene is driven by the E3 promoter and pA sequences, leaving more space for coding sequences.

To this end, the 2.7 kb EcoRI fragment from wt Ad5 10 containing the 5' part of the E3 region was cloned into the EcoRI site of pBluescript (KS<sup>-</sup>) (Stratagene). Next, the HindIII site in the polylinker was removed by digestion with EcoRV and HincII and subsequent re-ligation. The resulting clone pBS.Eco-Eco/ad5DHIII was used to delete the gp19K 15 coding region. Primers 1 (5'-GGG TAT TAG GCC AA AGG CGC A-3') and 2 (5'-GAT CCC ATG GAA GCT TGG GTG GCG ACC CCA GCG-3') were used to amplify a sequence from pBS.Eco-Eco/Ad5DHIII corresponding to sequences 28511 to 28734 in wt Ad5 DNA. Primers 3 (5'-GAT CCC ATG GGG ATC CTT TAC TAA GTT 20 ACA AAG CTA-3') and 4 (5'-GTC GCT GTA GTT GGA CTG G-3') were used on the same DNA to amplify Ad5 sequences from 29217 to 29476. The two resulting PCR fragments were ligated together by virtue of the new introduced NcoI site and subsequently digested with XbaI and MunI. This fragment was then ligated 25 into the pBS.Eco-Eco/ad5ΔHIII vector that was digested with XbaI (partially) and MunI generating pBS.Eco-Eco/ad5ΔHIII.Δgp19K. To allow insertion of foreign genes into the HindIII and BamHI site, an XbaI deletion was made in pBS.Eco-Eco/ad5ΔHIII.Δgp19K to remove the BamHI site in the Bluescript 30 polylinker. The resulting plasmid pBS.Eco-Eco/ad5ΔHIIIΔgp19KΔXbaI, contains unique HindIII and BamHI sites corresponding to sequences 28733 (HindIII) and 29218 (BamHI) in Ad5. After introduction of a foreign gene into these sites, either the

deleted XbaI fragment is re-introduced, or the insert is recloned into pBS.Eco-Eco/ad5 $\Delta$ HIII. $\Delta$ gpl9K using HindIII and for example MunI. Using this procedure, we have generated plasmids expressing HSV-TK, hIL-1 $\alpha$ , rat IL-3, luciferase or  
5 LacZ. The unique SrfI and NotI sites in the pBS.Eco-Eco/ad5 $\Delta$ HIII. $\Delta$ gpl9K plasmid (with or without inserted gene of interest) are used to transfer the region comprising the gene of interest into the corresponding region of pBr/Ad.Bam-rITR, yielding construct pBr/Ad.Bam-rITR $\Delta$ gpl9K  
10 (with or without inserted gene of interest). This construct is used as described *supra* to produce recombinant adenoviruses. In the viral context, expression of inserted genes is driven by the adenovirus E3 promoter.

Recombinant viruses that are both E1 and E3 deleted are  
15 generated by a double homologous recombination procedure as described above for E1-replacement vectors using a plasmid-based system consisting of:

- a) an adapter plasmid for E1 replacement according to the invention, with or without insertion of a first gene of  
20 interest,
- b) the pWE/Ad.AflIII-EcoRI fragment, and
- c) the pBr/Ad.Bam-rITR $\Delta$ gpl9K plasmid with or without insertion of a second gene of interest.

In addition to manipulations in the E3 region, changes  
25 of (parts of) the E4 region can be accomplished easily in pBr/Ad.Bam-rITR. Generation and propagation of such a virus, however, in some cases demands complementation *in trans*.

**Example 2****Generation of adenovirus serotype 5 based viruses with chimaeric fiber proteins**

5       The method described *infra* to generate recombinant adenoviruses by co-transfection of two, or more separate cloned adenovirus sequences. One of these cloned adenovirus sequences was modified such that the adenovirus serotype 5 fiber DNA was deleted and substituted for unique restriction  
10 sites thereby generating "template clones" which allow for the easy introduction of DNA sequences encoding for fiber protein derived from other adenovirus serotypes.

Generation of adenovirus template clones lacking DNA  
15 encoding for fiber

      The fiber coding sequence of adenovirus serotype 5 is located between nucleotides 31042 and 32787. To remove the adenovirus serotype 5 DNA encoding fiber we started with construct pBr/Ad.Bam-rITR. First a NdeI site was removed  
20 from this construct. For this purpose, pBr322 plasmid DNA was digested with NdeI after which protruding ends were filled using Klenow enzym. This pBr322 plasmid was then re-ligated, digested with NdeI and transformed into *E.coli* DH5 $\alpha$ . The obtained pBr/ $\Delta$ NdeI plasmid was digested with ScaI and  
25 SalI and the resulting 3198 bp vector fragment was ligated to the 15349 bp ScaI-SalI fragment derived from pBr/Ad.BamrITR, resulting in plasmid pBr/Ad.Bam-rITR $\Delta$ NdeI which hence contained a unique NdeI site. Next a PCR was performed with oligo nucleotides NY-up: 5'-CGA **CAT ATG** TAG  
30 ATG CAT TAG TTT GTG TTA TGT TTC AAC GTG-3' and NY-down: 5'-GGA GAC CAC TGC CAT GTT-3'. During amplification, both a NdeI (bold face) and a NsiI restriction site (underlined)

were introduced to facilitate cloning of the amplified fiber DNAs.

Amplification consisted of 25 cycles of each 45 sec. at 94°C, 1 min. at 60°C, and 45 sec. at 72°C. The PCR reaction  
5 contained 25 pmol of oligonucleotides NY-up or NY-down, 2mM dNTP, PCR buffer with 1.5 mM MgCl<sub>2</sub>, and 1 unit of Elongase heat stable polymerase (Gibco, The Netherlands). 10% of the PCR product was run on an agarose gel which demonstrated that the expected DNA fragment of ± 2200 bp was amplified.  
10 This PCR fragment was subsequently purified using Geneclean kit system (Bio101 Inc.). Then, both the constructs pBr/Ad.Bam-rITRΔNdeI as well as the PCR product were digested with restriction enzymes NdeI and SbfI. The PCR fragment was subsequently cloned using T4 ligase enzyme into  
15 the NdeI and SbfI digested pBr/Ad.Bam-rITRΔNdeI, generating pBr/Ad.BamRAΔFib. This plasmid allows insertion of any PCR amplified fiber sequence through the unique NdeI and NsiI sites that are inserted in place of the removed fiber sequence. Viruses can be generated by a double homologous  
20 recombination in packaging cells described *infra* using an adapter plasmid, construct pBr/Ad.AflIII-EcoRI digested with PacI and EcoRI and a pBr/Ad.BamRAΔFib construct in which heterologous fiber sequences have been inserted. To increase the efficiency of virus generation, the construct  
25 pBr/Ad.BamRAΔFib was modified to generate a PacI site flanking the right ITR. Hereto, pBr/Ad.BamRAΔFib was digested with AvrII and the 5 kb adenofragment was isolated and introduced into the vector pBr/Ad.Bam-rITR.pac#8 replacing the corresponding AvrII fragment. The resulting construct  
30 was named pBr/Ad.BamRAΔFib.pac.

Once a heterologous fiber sequence is introduced in pBr/Ad.BamRAΔFib.pac, the fiber modified right hand adenovirus clone may be introduced into a large cosmid clone

as described for pWE/Ad.AflIII-rITR in example 1. Such a large cosmid clone allows generation of adenovirus by only one homologous recombination making the process extremely efficient.

5

Amplification of fiber sequences from adenovirus serotypes.

To enable amplification of the DNAs encoding fiber protein derived from alternative serotypes degenerate oligonucleotides were synthesized. For this purpose, first  
10 known DNA sequences encoding for fiber protein of alternative serotypes were aligned to identify conserved regions in both the tail-region as well as the knob-region of the fiber protein. From the alignment, which contained the nucleotide sequence of 19 different serotypes  
15 representing all 6 subgroups, (degenerate) oligonucleotides were synthesised (see table 2). Also shown in table 2 is the combination of oligonucleotides used to amplify the DNA encoding fiber protein of a specific serotype. The amplification reaction (50 µl) contained 2 mM dNTPs, 25 pmol  
20 of each oligonucleotide, standard 1x PCR buffer, 1,5 mM MgCl<sub>2</sub>, and 1 Unit Pwo heat stable polymerase (Boehringer) per reaction. The cycler program contained 20 cycles, each consisting of 30 sec. 94°C, 60 sec. 60-64°C, and 120 sec. At 72°C. 10% of the PCR product was run on an agarose gel which  
25 demonstrated that a DNA fragment was amplified. Of each different template, two independent PCR reactions were performed after which the independent PCR fragments obtained were sequenced to determine the nucleotide sequence. From 11 different serotypes, the nucleotide sequence could be  
30 compared to sequences present in Genbank. Of all other serotypes, the DNA encoding fiber protein was previously unknown and was therefore aligned with known sequences from other subgroup members to determine homology i.e. sequence



divergence. Of the 51 human serotypes known to date, all fiber sequences, except for serotypes 1, 6, 18, and 26 have been amplified and sequenced.

5 Generation of fiber chimaeric adenoviral DNA constructs

All amplified fiber DNAs as well as the vector (pBr/Ad.BamRAFib) were digested with NdeI and NsiI. The digested DNAs was subsequently run on a agarose gel after which the fragments were isolated from the gel and purified  
10 using the Geneclean kit (Biol01 Inc). The PCR fragments were then cloned into the NdeI and NsiI sites of pBr/AdBamRAFib, thus generating pBr/AdBamRFibXX (where XX stands for the serotype number of which the fiber DNA was isolated). So far the fiber sequence of serotypes 5/ 7/ 8/ 9/ 10/ 11/ 12/ 13/  
15 14/ 16/ 17/ 19/ 21/ 24/ 27/ 28/ 29/ 30/ 32/ 33/ 34/ 35/ 36/ 37/ 38/ 40-S/ 40-L/ 41-S/ 42/45/ 47/ 49/ 51 have been cloned into pBr/AdBamRFibXX. From pBr/AdBamRFibXX (where XX is 5/ 8/ 9/ 10/ 11/ 13/ 16/ 17/ 24/ 27/ 30/ 32/ 33/ 34/ 35/ 38/ 40-S/ 40-L/ 45/ 47/ 49/ 51) a cosmid clone in pWE/Ad.AflIII-rITR (see example 1) was generated to facilitate efficient  
20 virus generaion. This cosmid cloning resulted in the formation of construct pWE/Ad.AflIII-rITR/FibXX (where XX stands for the serotype number of which the fiber DNA was isolated)

25

Generation of recombinant adenovirus chimaeric for fiber protein

To generate recombinant Ad 5 virus carrying the fiber of serotype 12, 16, 28, 40-L, 51, and 5, three constructs,  
30 pCLIP/luciferase, pWE/AdAflIII-Eco and pBr/AdBamrITR.pac/fibXX (XX = 12, 16, 28, 40-L, 51, and 5) were transfected into adenovirus producer cells. To generate recombinant Ad 5 virus carrying the fiber of 5/ 7/ 8/ 9/ 10/

11/ 12/ 13/ 14/ 16/ 17/ 19/ 21/ 24/ 27/ 28/ 29/ 30/ 32/ 33/  
34/ 35/ 36/ 37/ 38/ 40-S/ 40-L/ 41-S/ 42/45/ 47/ 49/ 51, two  
constructs pCLIP/luciferase and pWE/Ad.AflIII-rITR/FibXX were  
transfected into adenovirus producer cells.

5 For transfection, 2 µg of pCLIP/luciferase, and 4 µg of  
both pWE/AdAflIII-Eco and pBr/AdBamrITR.pac/fibXX (or in case  
of cosmids: 4 µg of pCLIP/luciferase plus 4 µg of  
pWE/Ad.AflIII-rITR/FibXX) were diluted in serum free DMEM to  
100 µl total volume. To this DNA suspension 100 µl 1x  
10 diluted lipofectamine (Gibco) was added. After 30 minutes at  
room temperature the DNA-lipofectamine complex solution was  
added to 2.5 ml of serum-free DMEM which was subsequently  
added to a T25 tissue culture flask. This flask contained  
2x10<sup>6</sup> PER.C6 cells that were seeded 24-hours prior to  
15 transfection. Two hours later, the DNA-lipofectamine complex  
containing medium was diluted once by the addition of 2.5 ml  
DMEM supplemented with 20% fetal calf serum. Again 24 hours  
later the medium was replaced by fresh DMEM supplemented  
with 10% fetal calf serum. Cells were cultured for 6-8 days,  
20 subsequently harvested, and freeze/thawed 3 times. Cellular  
debris was removed by centrifugation for 5 minutes at 3000  
rpm (room temperature). Of the 12.5 ml supernatant 3-5 ml  
was used to infect again PER.C6 cells (T80 tissue  
culture flasks). This re-infection results in full  
25 cytopathogenic effect (CPE) after 5-6 days after which the  
adenovirus is harvested as described above.

### Example 3

Production, purification, and titration of fiber chimaeric  
30 adenoviruses

Of the supernatant obtained from transfected PER.C6  
cells 10 ml was used to inoculate a 1 liter fermentor which

contained  $1-1.5 \times 10^6$  cells/ml PER.C6 that were specifically adapted to grow in suspension. Three days after inoculation, the cells were harvested and pelleted by centrifugating for 10 min at 1750 rpm at room temperature.

5 The chimaeric adenoviruses present in the pelleted cells were subsequently extracted and purified using the following downstream processing protocol. The pellet was dissolved in 50 ml 10 mM  $\text{NaPO}_4^-$  and frozen at  $-20^\circ\text{C}$ . After thawing at  $37^\circ\text{C}$ , 5.6 ml deoxycolate (5% w/v) was added after which the

10 solution was homogenated. The solution was subsequently incubated for 15 min at  $37^\circ\text{C}$  to completely crack the cells. After homogenizing the solution, 1875  $\mu\text{l}$  (1M)  $\text{MgCl}_2^-$  was added and 5 ml 100% glycerol. After the addition of 375  $\mu\text{l}$  DNase (10 mg/ml) the solution was incubated for 30 min at

15  $37^\circ\text{C}$ . Cell debris was removed by centrifugation at 1880xg for 30 min at room temperature without the brake on. The supernatant was subsequently purified from proteins by loading on 10 ml of freon. Upon centrifugation for 15 min at 2000 rpm without brake at room temperature three bands are

20 visible of which the upper band represents the adenovirus. This band was isolated by pipetting after which it was loaded on a Tris/HCl (1M) buffered cesium chloride block gradient (range: 1.2 to 1.4 g/ml). Upon centrifugation at 21000 rpm for 2.5 hours at  $10^\circ\text{C}$  the virus was purified from

25 remaining protein and cell debris since the virus, in contrast to the other components, does not migrate into the 1.4 g/ml cesium chloride solution. The virus band is isolated after which a second purification using a Tris/ HCl (1M) buffered continuous gradient of 1.33 g/ml of cesium

30 chloride is performed. After virus loading on top of this gradient the virus is centrifuged for 17 h at 55,000 rpm at  $10^\circ\text{C}$ . Subsequently the virus band is isolated and after the addition of 30  $\mu\text{l}$  of sucrose (50 w/v) excess cesium chloride

is removed by three rounds of dialysis, each round comprising of 1 h. For dialysis the virus is transferred to dialysis slides (Slide-a-lizer, cut off 10,000 kD, Pierce, USA). The buffers used for dialysis are PBS which are  
5 supplemented with an increasing concentration of sucrose (round 1 to 3: 30 ml, 60 ml, and 150 ml sucrose (50% w/v)/1.5 liter PBS, all supplemented with 7.5 ml 2% (w/v)  $\text{CaMgCl}_2$ ). After dialysis, the virus is removed from the slide-a-lizer after which it is aliquoted in portions of 25  
10 and 100  $\mu\text{l}$  upon which the virus is stored at  $-85^\circ\text{C}$ .

To determine the number of virusparticles per ml, 100  $\mu\text{l}$  of the virus batch is run on an high pressure liquid chromatograph (HPLC). The adenovirus is bound to the column (anion exchange) after which it is eluted using a NaCl  
15 gradient (range 300-600 mM). By determining the area under the virus peak the number of virus particles can be calculated. To determine the number of infectious units (IU) per ml present in a virus batch, titrations are performed on 911 cells. For this purpose,  $4 \times 10^4$  911 cells are seeded per  
20 well of 96-well plates in rows B, D, and F in a total volume of 100  $\mu\text{l}$  per well. Three hours after seeding the cells are attached to the plastic support after which the medium can be removed. To the cells a volume of 200  $\mu\text{l}$  is added, in duplicate, containing different dilutions of virus (range:  
25  $10^2$  times diluted to  $2 \times 10^9$ ). By screening for CPE the highest virus dilution which still renders CPE after 14 days is considered to contain at least one infectious unit. Using this observation, together with the calculated amount of virus volume present in these wells renders the number of  
30 infectious units per ml of a given virus batch. The production results i.e. virus particles per ml and IU per ml or those chimaeric adenoviruses that were produced, all with the luciferase cDNA as a marker, are shown in table 3.

**Example 4****Expression of receptors for Adenovirus 5 on Primary human fibroblasts**

5 To test whether for expression on primary fibroblasts for membrane molecules known to be involved in Ad5 infection, the presence of CAR, MHC class I, and  $\alpha_v$ -integrins was assayed on a flow cytometer. For this purpose  $1 \times 10^5$  HUVEC cells or HUVsmc were washed once with PBS/0.5% BSA after which the cells were pelleted by centrifugation for 5 min at 1750 rpm at room temperature. Subsequently, 10  $\mu$ l of a 100 times diluted  $\alpha_v\beta 3$  antibody (Mab 1961, Brunswick chemie, Amsterdam, The Netherlands), a 100 times diluted antibody  $\alpha_v\beta 5$  (antibody (Mab 1976, Brunswick chemie, 15 Amsterdam, The Netherlands), or 2000 times diluted CAR antibody was a kind gift of Dr. Bergelson, Harvard Medical School, Boston, USA (Hsu et al. 1988) was added to the cell pellet after which the cells were incubated for 30 min at 4°C in a dark environment. After this incubation, cells were 20 washed twice with PBS/0.5% BSA and again pelleted by centrifugation for 5 min at 1750 rpm room temperature. To label the cells, 10 ml of rat anti mouse IgG1 labeled with phycoerythrine (PE) was added to the cell pellet upon which the cells were again incubated for 30 min at 4°C in a dark 25 environment. Finally the cells were washed twice with PBS/0.5% BSA and analysed on a flow cytometer. The results of these experiments are shown in Figure 1. From the results it can be concluded that primary human fibroblasts do not express detectable levels of CAR or MHC-class I confirming 30 that these cells are difficult to transduce with an adenovirus which enters the cells via these molecules.

**Example 5****Adenovirus transduction of human Primary Fibroblasts**

Human primary fibroblasts were routinely maintained in Dulbecco's modified Eagles medium (DMEM) supplemented with 10% fetal calf serum. Fibroblasts tested were from different origins. Normal human fibroblasts were obtained from Coriell (GM09503), or were isolated from skin biopsies from healthy human individuals. In a first experiment,  $10^5$  fibroblasts were seeded in wells of 24-well plates. The next day cells were exposed to either 100, 500, or 1000 virus particles per cell of recombinant fiber chimaeric viruses carrying the fiber of serotype 9, 10, 11, 12, 13, 16, 17, 24, 27, 30, 32, 33, 35, 38, 40-S, 40-L, 45, 47, 49, or 51. In these experiments, the parent vector (fib5) was taken along as a reference. Forty-eight hours after the addition of virus, cells were washed twice with 1 ml PBS after which cells were lysed by adding 100  $\mu$ l of cell lysis buffer. Lysates were subsequently transferred to 96-well plates and stored at -20°C until luciferase activity measurement. Luciferase activity was determined using a bioluminescence machine, the luciferase assay kit from Promega (catalog no. E-1501) and the instructions provided by the manufacturer.

The results of the luciferase transgene expression measured in primary human fibroblasts after transduction with the panel of fiber chimeaic viruses is shown in figure 2. The results demonstrate that several fiber chimaeric viruses perform better on fibroblasts as compared to the parent vector (Ad5). These viruses carry the fiber from a subgroup B virus i.e. 11, 16, 35, and 51. Also, several, but not all, viruses carrying a fiber originating from subgroup D i.e. 9, 13, 17, 32, 38 seem better equipped for transducing fibroblasts. Also, the short fiber of

serotype 40 (40-S), a F-group virus performs better than Ad5. Clearly, based on luciferase expression the Ad5 vector carrying a fiber of serotype 16 looked most promising. In a next experiment, Ad5 was directly compared to Ad5.Fib16, Ad5.Fib35, and Ad5.Fib51 using green fluorescent protein (GFP) as a marker gene. This marker gene allows single cell analysis using a flow cytometer. Infection of fibroblasts using the GFP viruses was performed identical as described above. The results on GFP expression is shown in figure 3. These results confirm the results of the first experiment for the fiber chimaeric viruses Ad5.Fib16, Ad5.Fib35, and Ad5.Fib51.

In another experiment primary fibroblasts were isolated from 4 different donors and cultured. Subsequently, these cells were transduced in parallel with Adenoviral vectors. The vectors that were used in this case were recombinant Ad5 and Ad5.Fib51 (Adenovirus serotype 5 with a Fiber protein derived from Adenovirus serotype 51). Both recombinant vectors harbor a LacZ transgene. The dosages that were used for transduction were 0, 25, 50, 100, 250 and 500 virus particles per cell (vp/cell). Cells were incubated with the Adenoviruses for 1 hour and washed thereafter with fresh medium. Subsequently, cells were further incubated for 48 h after which the cells were stained for LacZ expression, and counted under a light microscope and compared to the non-transduced cells. Percentages of positive cells are indicated in Figure 4. This shows that there is a possible difference between different patient derived primary fibroblast batches that are used for adenoviral transduction using the two adenoviral vector batches that were used in this case. However, in general these results suggest strongly that Ad5.Fib51 is able to significantly transduce these cells better than the Ad5 vectors.

## SHORT DESCRIPTION OF THE FIGURES AND TABLES

Table 1: Association of different human adenovirus serotypes  
5 with human disease.

Table 2: Skin substitutes: The manufacturers are as follows:  
Alloderm: Life cell, Woodlands, Texas; Integra, Life  
sciences, Plainsboro, N.J.; Dermagraft-TC, Advanced tissue  
10 sciences, La Jolla, California; Apligraf, Organogenesis,  
Canton, Massachussetts; Composite cultured skin: Ortec  
international, New York. (from Singer et al. 1999)

Table 3: Production results of recombinant fiber chimaeric  
15 adenoviruses. Results are given in virus particles per  
milliliter as determined by HPLC.

Figure 1: Expression of CAR, MHC-class I, and av-integrins  
on primary fibroblasts. As a control for the antibodies  
20 PER.C6 cells were taken along.

Figure 2: Screening the fiber chimaeric viruses for the  
presence of viruses that are better suited for transduction  
of primary human fibroblasts. The dose used is 100 (grey  
25 bars), 500 (white bars), or 1000 (black bars) virus  
particles per cell. Luciferase activity is expressed in  
relative light units (RLU).

Figure 3: Flow cytometric analysis on fibroblasts transduced  
30 with 100 (white bars) or 1000 (black bars) virus particles  
of fiber chimaeric viruses carrying green fluorescent  
protein as a marker. Shown is the median fluorescence  
obtained after transduction with either Ad5 or the fiber



chimaeric viruses carrying the fiber of serotype 16, 35, or 51.

Figure 4: Primary fibroblasts derived from 4 different  
5 donors were cultured and transduced in parallel. The vectors  
used were Ad5 and Ad5.Fib51 carrying a LacZ transgene and  
different dosages (virus particles per cell) were used as  
indicated. Forty-eight hours after a virus exposure (which  
lasted for 1 h), the cells were stained for LacZ expression  
10 and compared to non-transduced plates.

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Table 1

	<u>Syndrom</u>	<u>Subgenus</u>	<u>Serotype</u>
5	Respiratory illness	A	31
		B	3, 7, 11, 14, 21, 34, 35, 51
		C	1, 2, 5, 6
		D	39, 42-48
		E	4
10	Keratoconjunctivitis (eye)	B	11
		D	8, 19, 37, 50
15	Hemorrhagic cystitis (Kidney)	B	7, 11, 14, 16, 21, 34, 35
		C	5
		D	39, 42-48
		C	2
		D	19, 37
20	Gastroenteritis	A	31
		B	3
		C	1, 2, 5
		D	28
		F	40, 41
25	CNS disease	A	12, 31
		B	3, 7
		C	2, 5, 6
		D	32, 49
		A	31
30	Hepatitis	C	1, 2, 5
		A	31
		B	3, 7, 11, 21
		D	30, 43-47
		A	18
35	Disseminated	D	9, 10, 13, 15 17, 20, 22-29, 33, 36, 38
		D	
	None	A	
		D	



Table 2

5	type of skin substitute /brand name	Components	Advantages	Disadvantages
10	<b>Epidermal</b>	Cultured autologous epidermal cells Cultured allogeneic Epidermal cells	Wide and permanent skin coverage Ready availability, no need for biopsy	2-3 week delay, high cost fragility, labor intensive use Temporary superficial coverage
15				
20	<b>Dermal</b>	Cryopreserved allogeneic Skin	Ready availability, use as base for cultured Epidermal cells	Need for procurement, potential disease transmission
25	<b>Alloderm</b>	Decellularized allogeneic Human skin	Ready availability, inert nature, use as base For epidermal grafts	Need for procurement, potential disease transmission
30	<b>Integra</b>	Bovine collagen with Chondroitin-6-sulphate	Ready availability, possible use of thin Autograft, reduced scarring	Need to excise wounds, risk of infection, high cost
35	<b>Combined Epidermal/ dermal</b>			
40	<b>Appligraf</b>	Bovine collagen, allogeneic Fibroblasts and epidermal Cells	Ready availability, no need for subsequent autografting	Limited viability
45	<b>Composite Cultured skin</b>	Collagen matrix substrate with fibroblasts and Epidermal cells	Ready availability, no need for subsequent autografting	Limited viability

Table 3

Adenovirus	Virus particles/ ml
Ad5Fib5	$2.2 \times 10^{12}$
Ad5Fib9	$4.9 \times 10^{11}$
Ad5Fib10	$5.5 \times 10^{11}$
Ad5Fib11	$1.1 \times 10^{12}$
Ad5Fib12	$4.4 \times 10^{12}$
Ad5Fib13	$1.1 \times 10^{12}$
Ad5Fib16	$1.4 \times 10^{12}$
Ad5Fib17	$9.3 \times 10^{11}$
Ad5Fib24	$1.0 \times 10^{12}$
Ad5Fib27	$3.0 \times 10^{11}$
Ad5Fib30	$7.1 \times 10^{11}$
Ad5Fib32	$2.0 \times 10^{12}$
Ad5Fib33	$1.5 \times 10^{12}$
Ad5Fib35	$2.0 \times 10^{12}$
Ad5Fib38	$5.8 \times 10^{11}$
Ad5Fib40-S	$3.2 \times 10^{10}$
Ad5Fib40-L	$2.0 \times 10^{12}$
Ad5Fib45	$2.8 \times 10^{12}$
Ad5Fib47	$2.6 \times 10^{12}$
Ad5Fib49	$1.2 \times 10^{12}$
Ad5Fib51	$5.1 \times 10^{12}$

CLAIMS

1. Use of a recombinant adenovirus having a tropism for human primary fibroblasts as a vehicle for delivering a nucleic acid of interest to a human primary fibroblast.
- 5 2. Use according to claim 1, in which said recombinant adenovirus is a chimaeric adenovirus.
3. Use according to claim 1 or 2, wherein said tropism is provided by at least a tropism determining part of an  
10 adenoviral fiber protein of a B-type or a D-type adenovirus.
4. Use according to claim 3, wherein said adenoviral fiber protein is derived from an adenovirus type 11, 16, 35, 51, 9, 13, 17, 32 and/or 38.  
15
5. Use according to claim 1 or 2, wherein said adenoviral fiber protein is derived from a short fiber protein of an adenovirus type 40.
- 20 6. Use according to claim 4, wherein said adenoviral fiber protein is derived from an adenovirus type 16 or a functional equivalent and/or homologue thereof.
7. Use according to any one of claims 1-6 wherein said  
25 nucleic acid of interest encodes a proteinaceous substance that improves angiogenesis and/or neovascularisation.
8. Use according to any one of claims 1-6 wherein said  
30 nucleic acid of interest is a MyoD gene and/or a Myogenin gene or a functional equivalent thereof.

9. Use according to any one of claims 2-8 wherein said chimaeric virus comprises an adenovirus 5 nucleic acid sequence.

5

10. Use according to claim 9, wherein said chimaeric virus comprises an adenovirus 5 genome, having at least a deletion in its E1 region where a nucleic acid of interest is inserted or can be inserted and a deletion in the fiber region which is replaced by a nucleic acid sequence having the desired tropism.

11. A gene delivery vehicle for delivering a nucleic acid of interest to a primary fibroblast, comprising a recombinant adenovirus having tropism for a primary fibroblast and a nucleic acid sequence encoding a proteinaceous substance which improves angiogenesis and/or neovascularisation as a nucleic acid of interest.

12. A gene delivery vehicle for delivering a nucleic acid of interest to a primary fibroblast, comprising a recombinant adenovirus having tropism for a primary fibroblast and a MyoD gene and/or a Myogenin gene or a functional equivalent thereof.

25

13. A gene delivery vehicle according to claim 11 or 12, wherein said tropism is provided by a nucleic acid sequence encoding at least a tropism determining part of a fiber protein of an adenovirus type B and/or adenovirus type D.

30

14. A gene delivery vehicle according to claim 11 or 12, wherein said tropism is provided by a nucleic acid sequence encoding at least a tropism determining part of a fiber

protein of an adenovirus type 11, 16, 35, 51, 9, 13, 17, 32 and/or 38.

15 15. A gene delivery vehicle according to claim 14, wherein said tropism is provided by a nucleic acid sequence encoding at least a tropism determining part of a fiber protein of an adenovirus type 16.

10 16. A gene delivery vehicle according to any one of claims 11-15 wherein said recombinant adenovirus is a chimaeric adenovirus.

15 17. A gene delivery vehicle according to claim 16, wherein said chimaeric virus comprises an adenovirus 5 genome, having at least a deletion in its E1 region where a nucleic acid of interest is inserted or can be inserted and a deletion in the fiber region which is replaced by a nucleic acid sequence having the desired tropism.

20 18. A gene delivery vehicle according to any one of claims 11-17 for use as a pharmaceutical.

25 19. Use of a gene delivery according to any one of claims 11 or 13-17 in the preparation of a medicament for woundhealing and/or skin transplantation.

20. A human primary fibroblast transduced with a gene delivery vehicle according to any one of claims 11, or 13-17.

30 21. A skin allograft comprising a human primary fibroblast or offspring thereof according to claim 20.

22. A method for improving allogeneic or autologous skin transplantation comprising transducing human primary fibroblasts with a gene delivery vehicle according to claim 11 or 13-17, preparing a skin graft with said human primary  
5 fibroblasts and applying said graft to a patient.

1/4

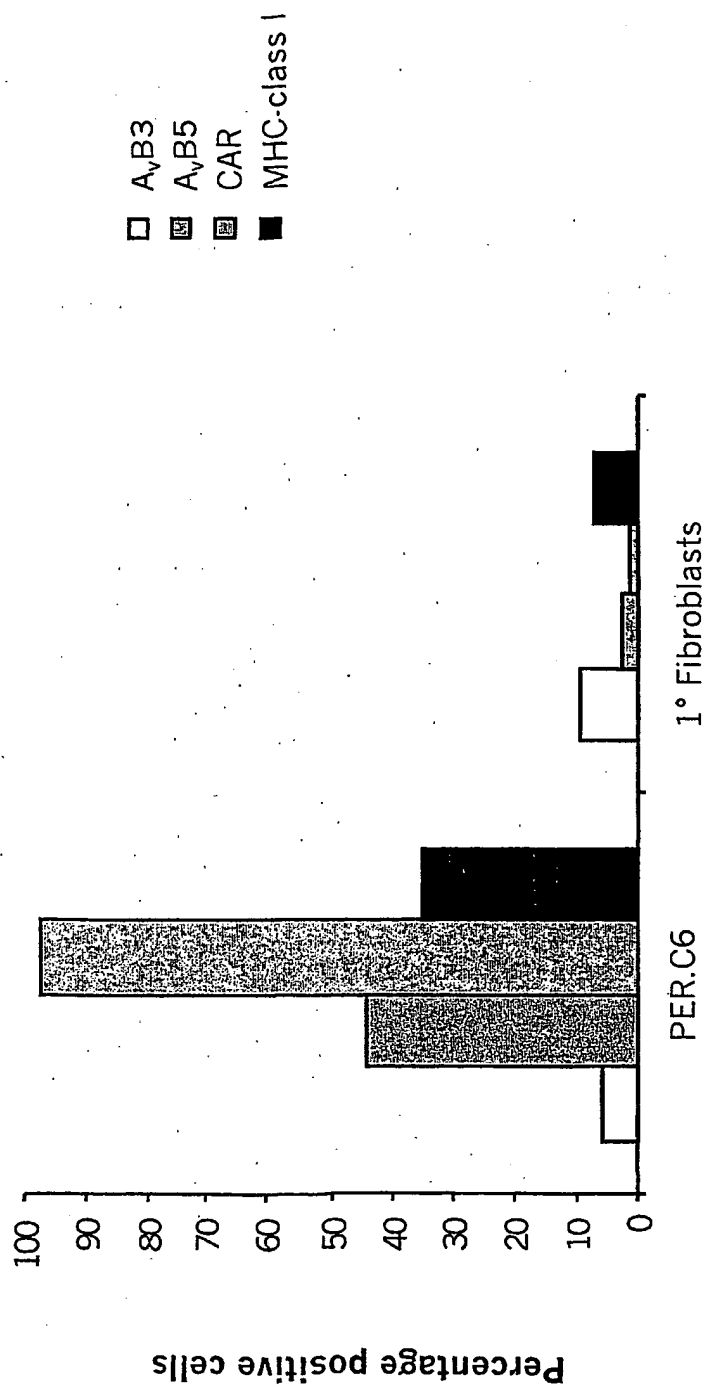
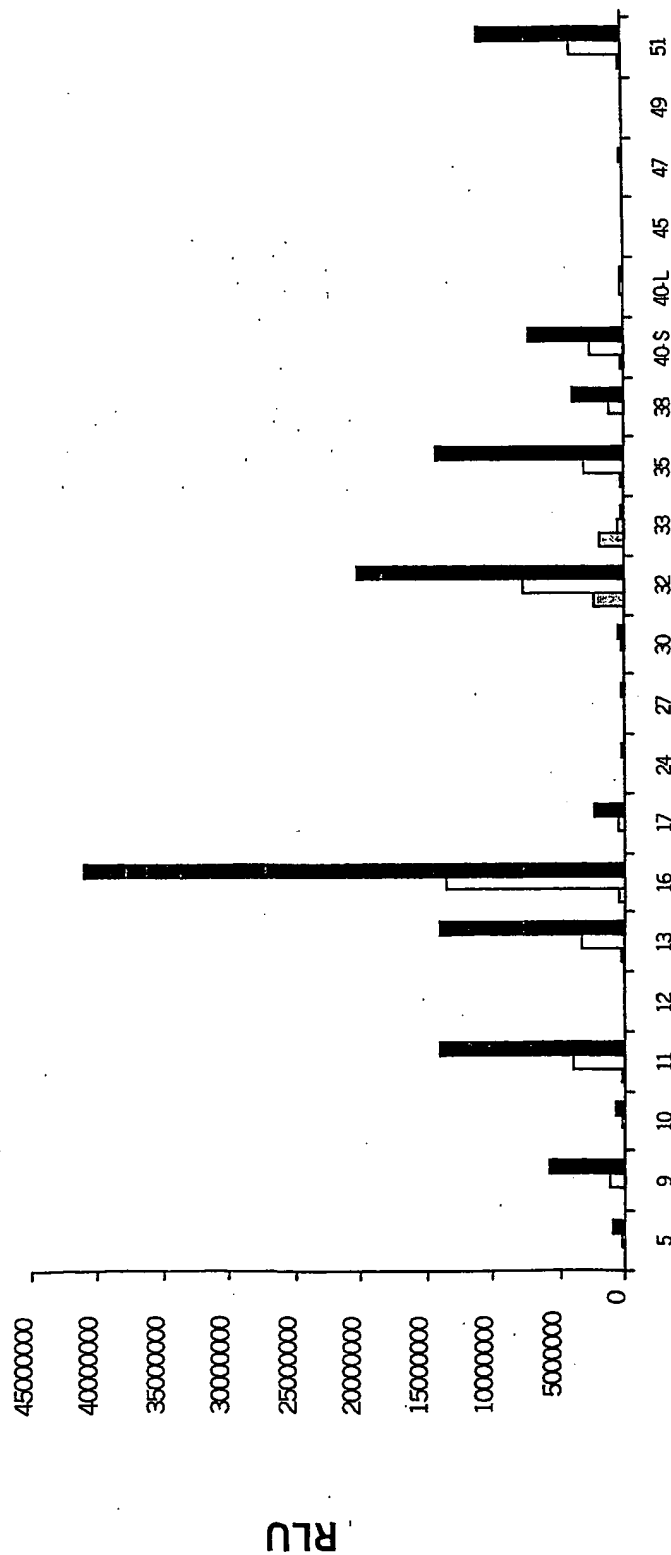


Fig. 1

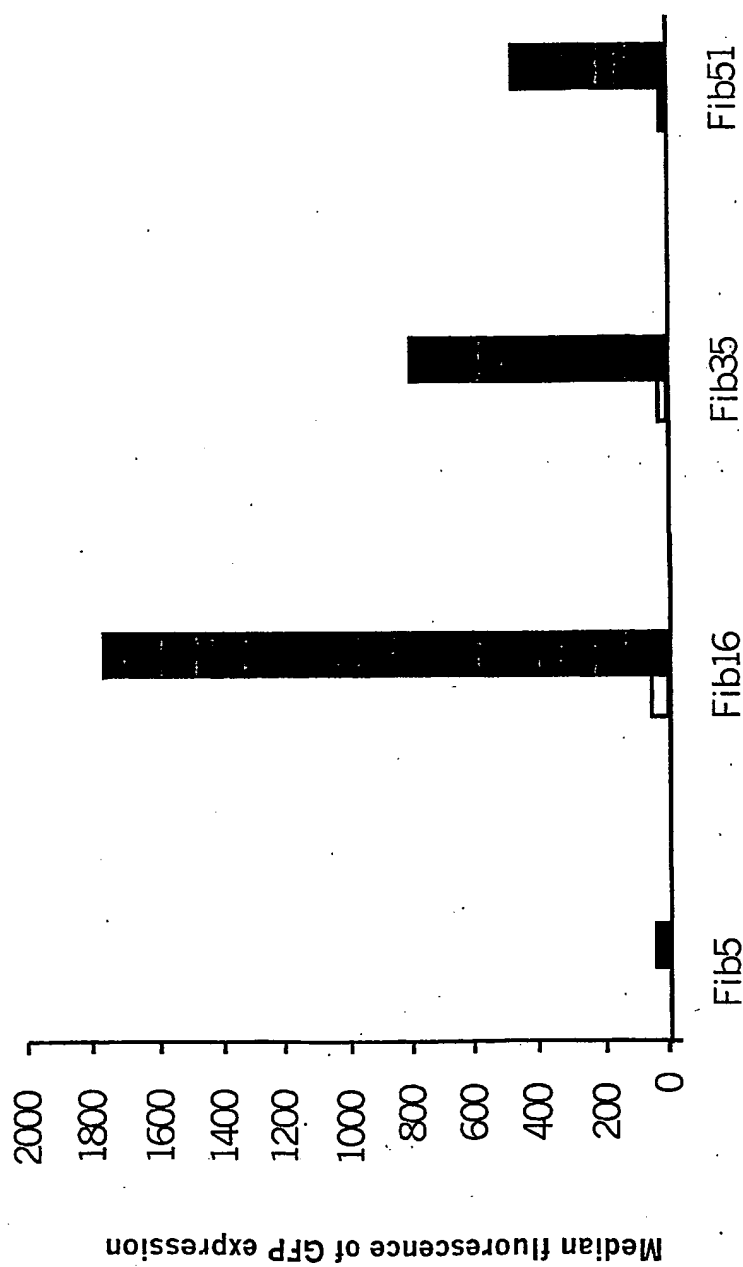
2/4



Recombinant fiber chimaeric adenovirus

Fig. 2





Recombinant fiber chimaeric adenovirus

Fig. 3

Transduction of Primary Fibroblasts (Patient variation)

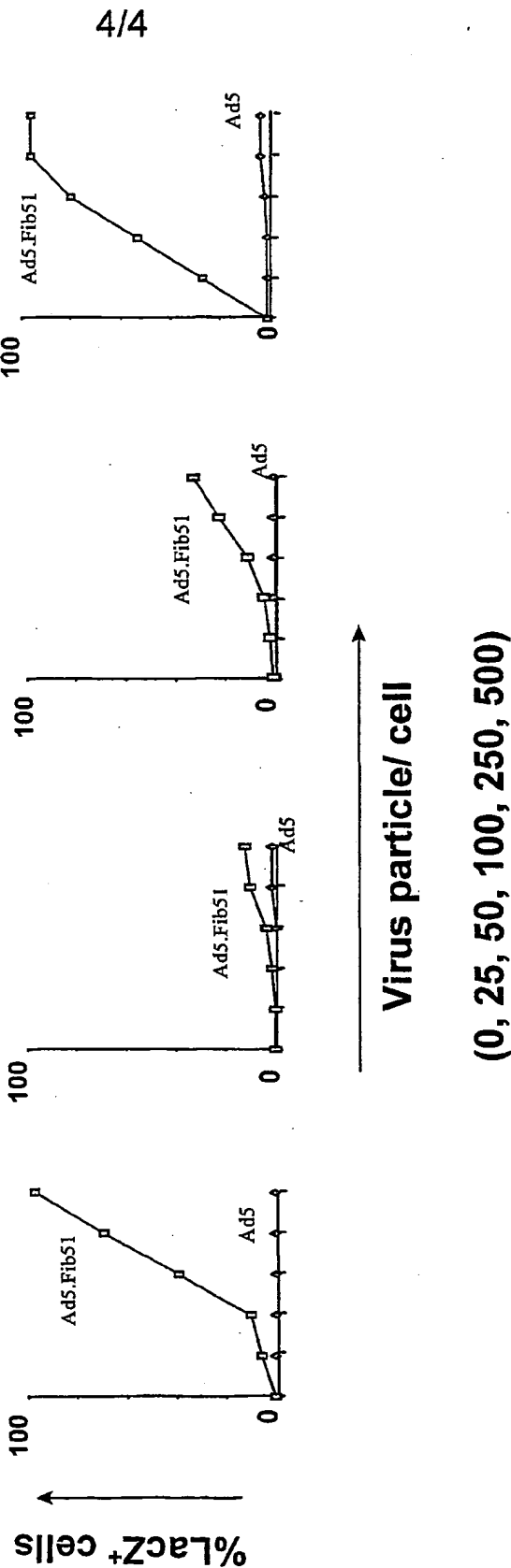


Fig. 4

## INTERNATIONAL SEARCH REPORT

International Application No  
PCT/NL 01/00402

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C07K14/075 C07K14/47 C12N15/86 C12N5/10 A61K48/00  
A61L27/60

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C07K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the International search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, MEDLINE, BIOSIS

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

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X	HIDAKA CHISA ET AL: "CAR-dependent and CAR-independent pathways of adenovirus vector-mediated gene transfer and expression in human fibroblasts" JOURNAL OF CLINICAL INVESTIGATION, US, NEW YORK, NY, vol. 103, no. 4, February 1999 (1999-02), pages 579-587, XP002139783 ISSN: 0021-9738	1-6, 9, 10
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☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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Date of the actual completion of the international search

2 October 2001

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## INTERNATIONAL SEARCH REPORT

Int'l Application No  
PCT/NL 01/00402

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X	WO 00 03029 A (INTROGENE BV) 20 January 2000 (2000-01-20)	1-6,9-18
Y	* the whole document, in particular page 9 lines 27-34, page 35 line 21, pages 42-43 example 7 and Table 6 *	7,8, 19-22
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